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Aerobic cultivation of high-oxygen-demanding microorganisms in the BioLector XT microbioreactor

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In this AppNote, we show how the BioLector XT microbioreactor can be used to cultivate microorganisms in a high-oxygen environment. Oxygen-enriched air up to 50% O_2 is used and the results are compared to experiments with regular atmospheric air gassing. The BioLector system's microfluidic module was used for precise open- and closed-loop feeding of substrate with a variety of different feeding strategies, while simultaneously offering control over the culture's pH throughout the experiment. The differences between experiments gassed with regular air and those with increased oxygen content illustrate the possibilities and advantages the BioLector XT microbioreactor offers by enabling cultivations under elevated oxygen concentrations.

Introduction

To optimize product yields, producer strains are genetically and metabolically engineered and fermentation processes are optimized and scaled to technical limits to achieve the highest space-time yields.[1] Among others, main considerations are the solubility, instability, and volatility of solid and gaseous substrates and products. Moreover, most media ingredients become inhibitory for biological systems at high concentrations. As a result, in a defined medium containing the maximum, non-inhibitive concentration of nutrients, Escherichia coli can be batch-cultivated only to a cell density of ~15 g_{cdw}/L. Therefore, well-designed media and feeding strategies are required to satisfy the increasing demands for energy, carbon, nitrogen, and further nutrients while preventing unwanted substrate-inhibition.[2, 3]

Another strong motivator for fed-batch cultivations is the so-called glucose overflow metabolism. Even under aerobic conditions, fast-growing organisms release fermentation products[4]; e.g., *Escherichia coli* produces acetate, *Bacillus subtilis* propionate, *Lactococcus lactis* lactate and *Saccharomyces cerevisiae* ethanol.[5] The formation of these by-products is (seemingly) wasteful as respiration leads to higher ATP yields than fermentation, and potentially inhibits several physiological properties of the culture itself.[2, 6] Nevertheless, the phenomenon is observed ubiquitously among many different organisms, including bacteria, fungi, and mammalian cells, which has resulted in a number of theories to explain the parallel respiration and fermentation, including repression of the TCA cycle,[7] more efficient proteome allocation,[4] and limited inner membrane space for the electron transport chain.[8]

However, for many applications these overflow metabolites are considered unwanted by-products, so microbial cultivations are usually performed under feeding conditions where specific nutrients – usually glucose – are limited. By doing so, various microorganisms have been grown to cell densities higher than 100 g_{cdw}/L, but some microorganisms are not suited for high cell-density cultivations (HCDC) and only capable of reaching concentrations of just a couple of grams per litre cell dry weight.[5]

Aerobic cultivations often depend on high oxygen transfer rates to prevent any limitations due to insufficient oxygen supply. For example, to achieve *E. coli* cell densities of 145 g_{cdw}/L, Horn et al. kept the dissolved oxygen above 20% air saturation (a.s.) throughout the cultivation.[9] To adequately achieve and maintain desired oxygen levels during HCDC, supplementing or substituting the air supply with pure oxygen might be necessary.[2] Dissolved oxygen concentrations below critical levels cause reduced specific growth rates, shifting metabolism and the aforementioned formation of unwanted metabolites such as acetate, lactate and ethanol, which results in lower cell densities and volumetric productivity in many different organisms.[10] However, gassing with increased oxygen concentrations could also promote higher leakage of reactive oxygen species (ROS).[11]

In both research and industry, a culture's dissolved oxygen (DO) concentration has also been used as a trigger for operating feed profiles. These closed-loop controllers are used to e.g., manipulate feeding rates based on on-line pH or DO values.[5] Feed-back controls based on DO values have been reported for a wide variety of organisms and products.[9, 12-17] They often rely on triggering additional substrate feeding based on a culture's rapidly increasing DO-signal caused by a lack of substrate.[18]

One organism of recent interest is *Vibrio natriegens* (alternatively known as *Pseudomonas natriegens* or *Beneckea natriegens*). It is the fastest growing, non-pathogenic organism known to date with reported generation times of only 9.4 minutes under optimal conditions.[1, 19] Similar to *E. coli*, it is a Gram-negative, facultatively anaerobic bacterium, but it has higher glucose and oxygen uptake rates to support its fast growth characteristics. It can grow on a wide variety of substrates and has been reported to secrete acetate as an overflow metabolite during aerobic cultivation on glucose.[20]

The fast growth rates make *V. natriegens* a potentially valuable chassis for many biotechnological applications and cloning procedures, and since 2016 it is accessible for genetic manipulation.[21, 22] Now, a collection of genetic tools has been established and applied to genetically engineer superior production and platform strains, but further fundamental knowledge is essential for future metabolic engineering strategies.[20]

In 2021, the first fed-batch HCDC with *V. natriegens* was reported with biomass yields up to 55 g_{cdw}/L. During fed-batch cultivations, oxygen uptake rates of up to 500 mmol * L^{-1} * h^{-1} were reported, which required aeration rates of up to 2 vvm with enriched air containing 60% oxygen to maintain the DO at 40% a.s. during the cultivation.

The BioLector XT microbioreactor is a powerful platform to further research and optimize these high-oxygen demanding cultivation strategies and processes, as it is capable of parallel fed-batch cultivations in µL-scale, precisely controlling feed rates per well. Furthermore, the sophisticated gassing lid gives superior control over gassing composition and flow rates. Oxygen enriched air up to 100% oxygen can be used to provide the cultures with sufficient oxygen to enable high oxygen transfer rates, especially when FlowerPlate microtiter plates are used.

Methods

Vibrio natriegens

Axenic *Vibrio natriegens*[23] was obtained from the DSMZ-German Collection of Microorganisms and Cell Cultures GmbH from the Leibniz Institutes (DSMZ Number 759). Furthermore, *Escherichia coli* BL 21(DE3) was used.

Cultivation medium and feed solution

Vibrio natriegens was cultivated in an adapted recipe of VN minimal medium,[1] with a set pH value of 7.5. The medium contained (L^{-1}): 42 g 3-(*N*-morpholino) propanesulfonic acid (MOPS), 5 g $(NH_4)_2SO_4$, 15 g NaCl, 1 g KH_2PO_4, 1 g K_2HPO_4, 0.25 g $MgSO_4$, 0.01 g $CaCl_2$, 16.4 mg $FeSO_4$ *7 H_2O, 10 mg $MnSO_4$ * H_20, 0.3 mg $CuSO_4$ * 5 H_20, 1 mg $ZnSO_4$ * $7H_2O$, 0.02 mg $NiCl_2$ * 6 H_2O, and was supplemented with an autoclaved glucose solution to a final concentration of 10 g * L^{-1} before inoculation. This recipe was also used for the incubation of pre-cultures in shake flasks.

For fed-batch cultivations, an adapted recipe of a feed solution allowing for HCDC[19] was used consisting of (L^{-1}): 500 g glucose, 20 g $(NH_4)_2SO_4$, 15 g NaCl, 20 g KH_2PO_4, 20 g K_2HPO_4, 5 g $MgSO_4$, 0.01 g $CaCl_2$, 16.4 mg $FeSO_4$ * 7 H_2O, 10 mg $MnSO_4$ * H_20, 0.3 mg $CuSO_4$ * 5 H_20, 1 mg $ZnSO_4$ * $7H_2O$, and 0.02 mg $NiCl_2$ * 6 H_2O.

E. coli was cultivated in Wilms-MOPS medium containing (L^{-1}): 10.5 g 3-(*N*-morpholino) propanesulfonic acid (MOPS, $C_7H_{15}NO_4S$), 3 g K_2HPO_4, 5 g $(NH_4)_2SO_4$, 0.5 g NH_4Cl, 2 g Na_2SO_4, 0.5 g $MgSO_4$ * 7 H_2O, 0.01 g thiamine hydrochloride ($C_{12}H_{17}ClN_4OS$ * HCl), 0.54 mg $ZnSO_4$ * 7 H_2O, 0.31 mg $CuSO_4$, 0.3 mg $MnSO_4$ * H_20, 41.76 mg $FeCl_3$ * 6 H_2O, 33.39 mg Titriplex III ($C_{10}H_{14}N_2Na_2O_8$ * 2 H_2O), 0.54 mg $CoCl_2$ * 6 H_2O, 1.98 mg $CaCl_2$ * 2 H_2O, and 20 g * L^{-1} glucose. For fed-batch cultivations, a feed solution was used containing 417 g * L^{-1} glucose, 42 g * L^{-1} $(NH_4)_2SO_4$, and 4.2 g * L^{-1} NH_4Cl.

Both *V. natriegens* and *E. coli* cultivations were designed with a one-sided pH-control with the help of 5 mol * L^{-1} NaOH, supplied to the cultures from the reservoir wells of MTP row B. Row A was used for the glucose-rich, fed-batch feeding solutions (microfluidic MTP geometry can be found in Figure 2).

Precultures in shake flasks

The main experiments in the BioLector XT microbioreactor were seeded from precultures incubated at 37°C and shaken at 280 rpm (25 mm orbital shaker) for several hours. *E. coli* precultures were grown in Wilms-MOPS as described above, except for having an increased MOPS-concentration of 42 g * L^{-1}. For *Vibrio natriegens*, the composition of the VN medium was unaltered. For both, 15 mL medium was inoculated from freshly thawed cryocultures and then cultivated in 250 mL shake flasks with cotton plugs.

The *E. coli* and *V. natriegens* precultures were incubated for 4 and 3 hours respectively to ensure exponential growth. Then, the cultures' OD_{600} was determined to calculate the volume required for inoculation.

Cultivation in the BioLector XT system

The experiments were done on the BioLector XT microbioreactor, equipped with a microfluidic module. Cultivations were conducted in Gen2 Microfluidic FlowerPlate microtiter plates (part no.: M2P-MTP-MF32C-BOH1), sealed with gas permeable sealing foils suited for microfluidic experiments (part no.: M2P-F-RSMF32-1). For real-time evaluation of the key cultivation parameters the following filter modules were installed: Biomass (ID: 501), pH (HP8, ID: 502) and DO (PSt3, ID: 503). The innovative BioLector XT system's gassing lid[24] allows for precise control over gassing composition – between 0 to 100% oxygen or up to 12% carbon dioxide – and flowrate, whereby the FlowerPlate microtiter plate ensures maximum (oxygen) transfer rates. Here, experiments were conducted using normal air (21% oxygen) and with increased oxygen concentrations up to 50%, both at a fixed flowrate of 50 mL * $minute^{-1}$. For the experiments with increased oxygen concentrations, the O_2 *up-regulation* gassing mode was used, and the oxygen content was gradually ramped up during the first hour after which it was kept constant at 50%.

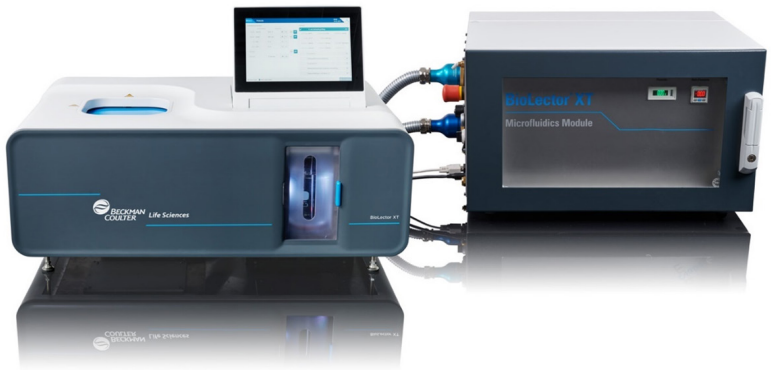

Figure 1. BioLector XT system equipped with the microfluidic module.

For *V. natriegens*, a starting OD_{600} of 0.1 was set by inoculation from the preculture. *E. coli* was seeded at OD_{600}=0.5. All cultivation wells (rows C to F) had a starting volume of 800 µL, and the FlowerPlate microtiter plate used has a maximum culture filling volume of 1400 µL at a shaker frequency of 1200 rpm. The maximum volume for the reservoir wells (row A and B) is 1800 µL, and were filled accordingly for the glucose feed in row A. In row B, 1200 µL base source was added. None of the reservoir wells ran empty during the presented cultivations in this application note.

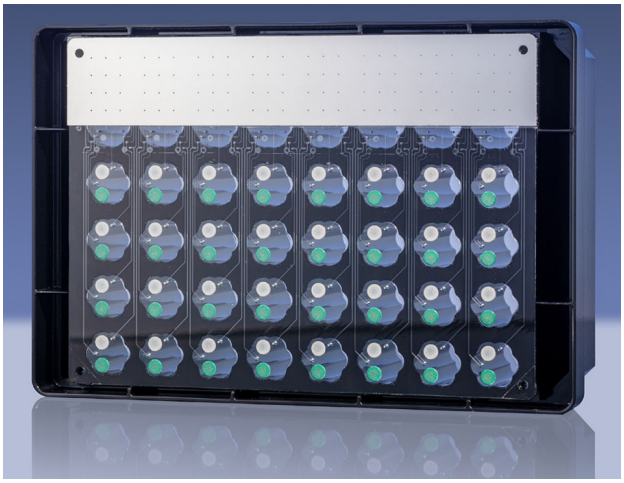

Figure 2. Gen2 Microfluidic FlowerPlate viewed from underneath. The lower four rows (C to F) consist of 32 cultivation wells, each containing 2 optodes attached to the bottom for optical pH and DO measurements. The top 2 rows – which are (partially) covered here by the deflexion limiter – are used as reservoir wells. Every reservoir well is connected to the four cultivation wells in the corresponding column by microfluidic channels running between the wells.

The pH-setpoint for all *E. coli* cultivations was set at 7.2 (pre-programmed BioLection software PI setting: *Medium*) and the one-sided pH-control was activated after 1 hour to allow the system some time to settle immediately after the experiment's start. The setpoint for the cultivations with *V. natriegens* was 7.5. Due to the high salt content of the VN minimal medium, using a custom pH-calibration dataset was necessary.[25] To better accommodate the fast growth characteristics of *V. natriegens*, custom PI settings were set: *Min. volume*: 0.1 µL * $cycle^{-1}$, *Max. volume*: 15 µL * $cycle^{-1}$, *P-band*: 0.2, *Integrator*: 1, *Start volume*: 0.2 µL, *Deadband*: 0.05.[26]

All glucose feeds were started after 4 hours for *E. coli*, and after 3.5 hours for *V. natriegens*. Based on (prior) batch cultivations under similar circumstances, the glucose supplemented to the media before inoculation should (almost) be depleted at this point. The time-triggered activation also allowed for good reproducibility and comparability between experiments with air gassing and increased oxygen gassing.

Several feeding profiles and strategies were set across the microtiter plate (MTP): For several wells, no glucose feeding was activated to achieve a pH-controlled batch cultivation. For the fed-batch cultivations, both continuous and signal-triggered feeding profiles were designed. The continuous feed rate strategies can be managed accurately in the BioLection software using the following formula where parameters A, B, C and D can all be selected independently:

$$\frac{dV}{dt} = A+B*t+C*e^{D*t}$$

For this application note, only constant and linearly increasing feeding rates were applied, so for parameter C and D a value of 0 was used for all strategies. The set values for A and B can be found in Table 1. The signal triggered feed was designed to pump 4 µL of glucose feeding solution into the cultivation every time the measured dissolved oxygen concentration would be higher than 6%. To compensate for unequal amounts of glucose feed or base added to the cultures, all online biomass measurements were corrected for their actual cultivation volume.

E. coli

Strategy	A [µL*h^{-1}]	B [µL*h^{-2}]
Constant	18	0
Linear	10	2

V. natriegens

Strategy	Pump volume [µL]
Signal triggered	4

Table 1. Set continuous feeding strategies for *E. coli* and *V. natriegens*.

Results

Improved *E. coli*-performance with oxygen-enriched air

The comparison of the *E. coli* fed-batch cultivations with regular and with oxygen-enriched air demonstrates the broader working range the O_2-up gassing mode adds to the microbioreactor and shows how it affects the cultivation course. The results of the *E. coli* cultivations with the continuous, constant feeding rate are shown in Figure 3.

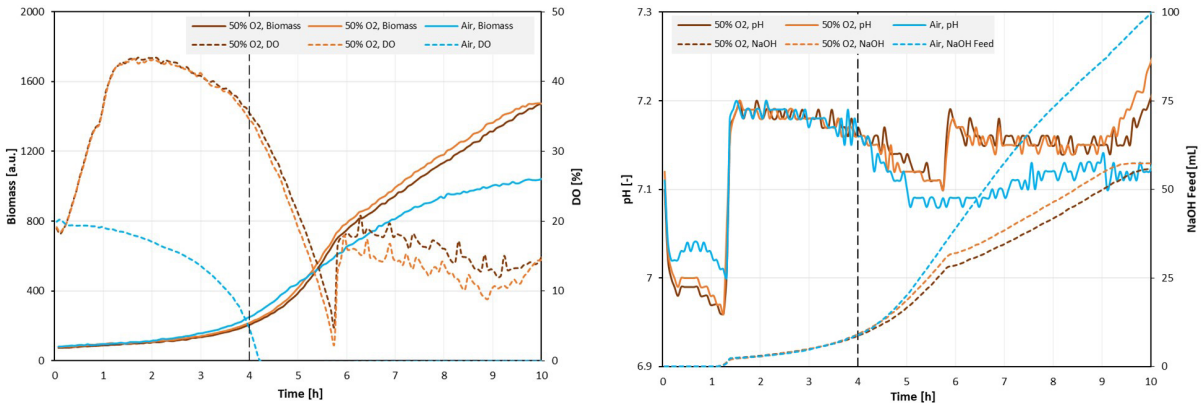

Figure 3. Fed-batch *E. coli* cultivations (37°C, 1200 rpm) with a continuous, constant feed rate of 18 µL * h^{-1}; gassing either with air (blue), or 50% O_2 (orange) in the BioLector XT microbioreactor. The left graph shows the online-measured biomass (left axis, solid lines) and DO (right axis, dashed lines), the right graph the pH (left axis, solid lines) and the volume of NaOH fed throughout the experiment (right axis, dashed lines). The dashed, vertical line indicates when the glucose feeding was started.

The dissolved oxygen course in the left graph in Figure 3 clearly shows the effect gassing with enriched oxygen has on the cultivation. The gradually increasing oxygen concentration in the inlet gas results in a gradually increasing DO signal during the first hour. Afterwards, the DO starts to drop in all cultures as *E. coli* grows aerobically in the presence of glucose. After 4 hours, glucose feeding is started and not long afterwards, the gassing with regular air no longer provides sufficient oxygen to the cultures. This leads to an oxygen-limitation throughout the remaining experiment. However, with the O_2 up-regulation the cultures are provided with sufficient oxygen to grow exponentially during the first 5.75 hours. After the prolonged exponential growth phase compared to the air-gassed cultivation, a linear growth phase is observed. Here, most likely, the constant glucose feeding rate is no longer sufficient to allow for exponential growth; and the culture grows glucose-limited throughout the rest of the experiment. Nevertheless, the resulting biomass concentrations are higher compared to the air-gassed (and oxygen-limited) cultivation.

The right graph shows the pH and total volume of NaOH used for pH-control during these experiments. During the first 4 hours the courses are highly comparable, but the effects of the oxygen limitation are observed as the fed-batch phase is started. The oxygen-deprived culture requires higher amounts of alkali, nonetheless the pH drops circa 0.1 below the setpoint after oxygen limitation occurs. Most likely, the (anaerobic) overflow metabolism results in higher production of secondary metabolites – like acetate – compared to the non-oxygen-limited cultivations which acidifies the culture.

For the *E. coli* cultivations with linear feeding profiles, very similar results were observed (Figure 4) as seen with the constant feed rates.

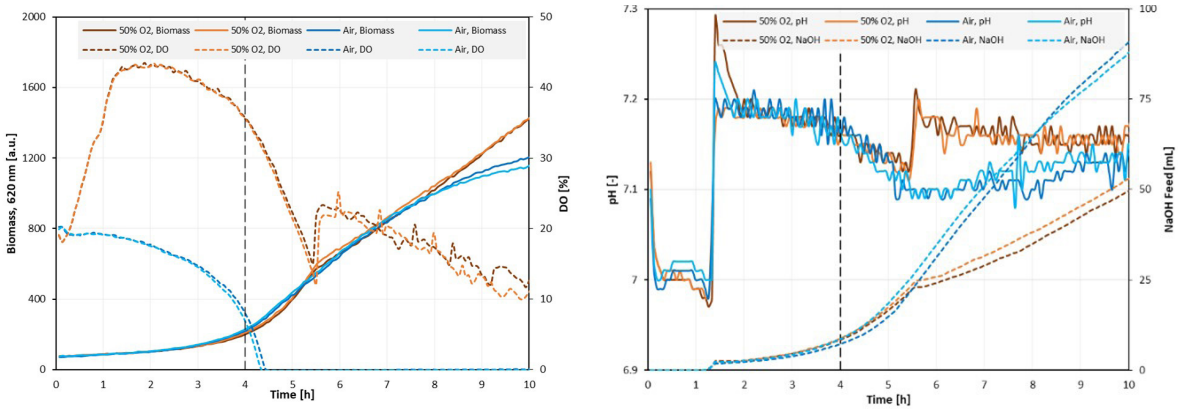

Figure 4. Fed-batch *E. coli* cultivations (37°C, 1200 rpm) with a continuous, linear feed rate of 10 µL * h^{-1} + 2 µL * h^{-2}; gassing either with air (blue), or 50% O_2 (orange) in the BioLector XT microbioreactor. The left graph shows the online-measured biomass (left axis, solid lines) and DO (right axis, dashed lines), the right graph the pH (left axis, solid lines) and the volume of NaOH fed throughout the experiment (right axis, dashed lines). The dashed, vertical line indicates when the glucose feeding was started.

Again, the cultures gassed with atmospheric air become oxygen-limited shortly after the glucose feed started whereas gassing with enriched oxygen prevents this from happening. Compared to Figure 3 however, the DO signal in the oxygen-enriched cultures does not drop as low and reaches a local minimum slightly earlier (after ca. 5.5 hours). This can be explained by the difference in the feeding profile between these cultivations. The first 4 hours after the feed is started, the constant feeding profile has a higher feed rate than the linear profile, which prolongs the exponential growth phase before glucose availability becomes the limiting factor. Like in the constantly fed wells, the oxygen-limited growth on glucose leads to higher volumes of NaOH fed to the culture, which demonstrates the metabolic differences occurring between aerobic and anaerobic growth of *E. coli*, and thus the effects gassing with enriched oxygen has on the cultivation.

Signal-triggered feeding strategy with *V. natriegens*

For the *Vibrio natriegens* cultivation, a feeding strategy to aim for high glucose consumption rates without forcing the culture into an oxygen-limitation was tested: signal-triggered glucose feeding based on oxygen availability in the culture medium. This should – when set correctly – prevent oxygen-limitation at a (near) maximum glucose feeding rate. To do so, the trigger for the glucose feed was set at DO >6%, and 4 µL of the glucose feed solution was added to the culture per feeding event. Cultivations were performed with air gassing and with oxygen enriched gassing, which is expected to enhance cultivation results because glucose feeding is triggered by oxygen availability. The results of these cultivations can be found in Figure 5.

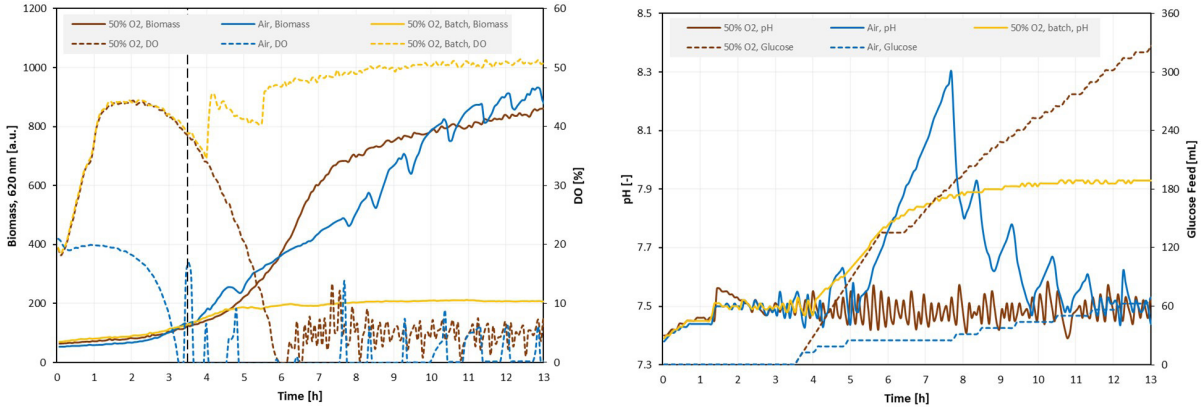

Figure 5. Fed-batch *V. natriegens* cultivations (37°C, 1200 rpm) with a DO signal-triggered feed with a pump volume of 4 µL; gassing either with air (blue), or 50% O_2 (brown) in the BioLector XT microbioreactor. In yellow, a batch cultivation gassed with enriched oxygen is depicted. One cultivation well per condition displayed. The left graph shows the online-measured biomass (left axis, solid lines) and DO (right axis, dashed lines), the right graph the pH (left axis, solid lines) and the volume of the glucose feed added throughout the experiment (right axis, dashed lines). The dashed, vertical line indicates when the glucose feeding was started.

The graphs in Figure 5 show a clearly distinct cultivation course when oxygen enriched air is used. During the initial batch mode, the cells already consume enough glucose to become oxygen-limited when no additional oxygen is applied to the culture. After four hours, the initial glucose is used up completely, as can be seen from increasing DO- and pH-values in the batch cultivation data (yellow data in Figure 5). The glucose feeding strategy was activated after 3.5 hours, and at that moment both the normal and enriched air cultivations had a DO-value larger than 6%, which triggered the immediate start of the glucose feed in both cultivations. Therefore, during the first ~ 15 minutes, the feed is identical for both conditions, but with regular air gassing, the DO-signal quickly drops below 6%, interrupting the glucose feed as the feeding trigger is no longer fulfilled.

For the cultivation with 50% oxygen, the increased oxygen content keeps the DO-signal above 6% for roughly 5.75 hours. Activation of the glucose feed after 3.5 hours prolongs the exponential growth phase compared to the batch cultivation and eventually results in an oxygen limitation roughly 6 hours after the experiment was started. The glucose feeding is stopped until the DO-value gets above the 6% threshold again after 6.5 hours. This results in a linear growth rate, strongly correlating to the glucose feed rate for the remaining experiment time, during which the DO-triggered glucose feed works as intended: Whenever the DO-signal supersedes the trigger threshold, glucose is added to the culture, which results in a decreasing DO-signal due to the aerobic metabolism on glucose. After glucose depletion the dissolved oxygen concentration increases again, which leads to another feeding event.

This feeding loop is repeated over 30 times and almost 200 µL of glucose feed was added to the cultivation in the remaining 6.5 hours, but no single feeding event resulted in a complete depletion of oxygen in the liquid phase. This shows that the DO-triggered feeding strategy was successfully incorporated with the help of the high oxygen transfer rates achieved by gassing with enriched oxygen in FlowerPlate microtiter plates and results in a tight control over glucose feeding rates based on online measurements. Also, the combination of (near-)continuous glucose feeding without inducing oxygen limitation prevents a metabolic shift to the consumption of secondary metabolites. This too allows for a good control over the culture's pH throughout the entire cultivation.

Compared to the cultivation gassed with normal air, more than five times more glucose feed was added. This is partially due to the oxygen limitation in the air-gassed cultivation quickly after the feed was started, which pauses the feed; but the effect can be seen throughout the whole experiment as (longer) periods of complete oxygen depletion occur in the air-gassing cultivation after each feeding pulse. The resulting feeding profile is reflected in the erratic course of the biomass, where a rapid increase in signal can be seen directly after each feeding event. Additionally, during the oxygen-limited phases the pH increases, which indicates that the organism temporarily grows on an acid produced as an overflow metabolite (like acetate) as a similar pH-increase can be seen in the batch cultivation after glucose depletion. However, the culture seems to switch back to glucose as the biomass signal increases while the pH decreases. This shift in metabolism seems to repeat itself at least seven times, and results in fluctuant pH-values well off the setpoint and a very opportunistic – and difficult to control – cultivation course when gassed with normal air.

Despite the lower glucose feeding in the culture gassed with regular air, the final biomass concentration of *V. natriegens* seems to be highly comparable between the two fed-batch cultivations shown. On one hand, the consumption of the secondary metabolite during the oxygen-limited phases could compensate for the lower glucose feeding as the oxygen-enriched cultures do not appear to make the diauxic shift in the shown cultivations. Nevertheless, the culture gassed with oxygen-enriched air receives a lot more glucose and the supplied glucose appears to be consumed quickly based on the decreasing oxygen concentration after glucose-feeding. The relatively low biomass formation might indicate a low glucose-to-biomass yield compared to the air-gassed cultivation and likely a higher formation rate of (by-)products like overflow metabolites.

In general, the DO-triggered fed-batch strategy is incorporated correctly in both cultivations. And though the shown cultivation with enriched oxygen was not optimized for high biomass production, it did allow for high glucose uptake rates without oxygen limitation and shows a more controlled and predictable cultivation course without the shifting metabolism seen in the air-gassed culture. In conclusion, this system demonstrates the impact of oxygen-enriched gassing on microbial cultivations that can further advance research by implementing the diverse and precise cultivation strategies provided by the BioLector XT system.

Conclusion

In this application note, the potential of the BioLector XT microbioreactor to research and optimize aerobic cultivation processes is discovered. The combination of the microfluidic module with the O_2-up gassing mode is a powerful tool to investigate different feeding strategies while preventing (harmful) oxygen-limitation, and it can help drive for optimal process parameters to enable higher cell densities and productivity. By using FlowerPlate microtiter plates and elevated oxygen concentrations, high-oxygen consuming organisms like *E. coli* and *V. natriegens* could be provided sufficiently with oxygen to prevent complete oxygen deprivation and improve culture performance and process control.

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